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13. ABSTRACT (Maximum 200 Words)

We have previously shown that the cell adhesion molecule β-catenin can form a complex with the androgen receptor (AR) and modulate its transcription. The cross talk between β-catenin and AR signaling can play an important role in AR transcriptional in prostate cancer progression. The objective of this study is to determine the mechanisms by which β-catenin modulates AR signaling. We show that β-catenin can increase AR transcriptional activation, and can also restore the transcriptional activity of loss-of-function AR (507-919). We find that amino acid 716 is required for the interaction between AR and β-catenin. To better understand AR-dependent transcription in vivo and the dynamics of AR/beta catenin interaction at gene promoters we used chromatin immunoprecipitation assays (ChIP assay) for the prostate-specific antigen (PSA) gene, the best-characterized androgen-responsive gene in the prostate gland. The results indicate that the dynamic loading of β-catenin to the PSA promoter and enhancer shares the same pattern with AR in presence of dihydrotestosterone (DHT) and this loading is inhibited by the AR competitive antagonist, bicalutamide (Casodex®). In the absence of DHT, bicalutamide can recruit both AR coactivators and corepressors to PSA gene.

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A. Introduction

Androgen-independence is a critical and pervasive late event in the progression of metastatic prostate cancer. Most androgen-independent prostate tumors continue to express androgen receptor (AR) suggesting that maintaining a functional AR signaling pathway is favorable for cell growth despite castrate levels of testosterone (1). In many instances, the cells bypass the requirement for physiologic levels of testosterone by enhancing transcriptional activation by the AR. However, the role of AR in prostate cancer progression is not fully understood. We have previously shown that the oncogene β-catenin is mutated in human prostate cancer (1,2). β-Catenin binds E cadherin at the cell membrane, playing a pivotal role in cell-cell adhesion. β-Catenin is also an essential signaling molecule in the Wnt growth pathway were it acts as a transcriptional coactivator for the T cell factor (TCF) family of proteins (3,4). Wnt signaling plays important roles in cell proliferation, differentiation and oncogenesis. We have shown that β-catenin can also form a complex with the androgen receptor (AR) enhancing transcription from androgen response elements (ARE), can change the sensitivity of the receptor for ligands and can relieve the suppression of antiandrogens on androgen-dependent transcription(5). The cross talk between β-catenin and AR signaling can play a role in prostate cancer progression. The objective of this study is to determine the mechanism by which β-catenin modulates AR signaling. *Our hypothesis is that there is a direct molecular interaction between β-catenin and the C-terminus region of AR.* We have based this hypothesis on our preliminary data showing co-precipitation of β-catenin with AR and results from reporter gene assays showing that deletion of C-terminal region of AR abolishes β-catenin' effect on AR-dependent transcription.

B. Body

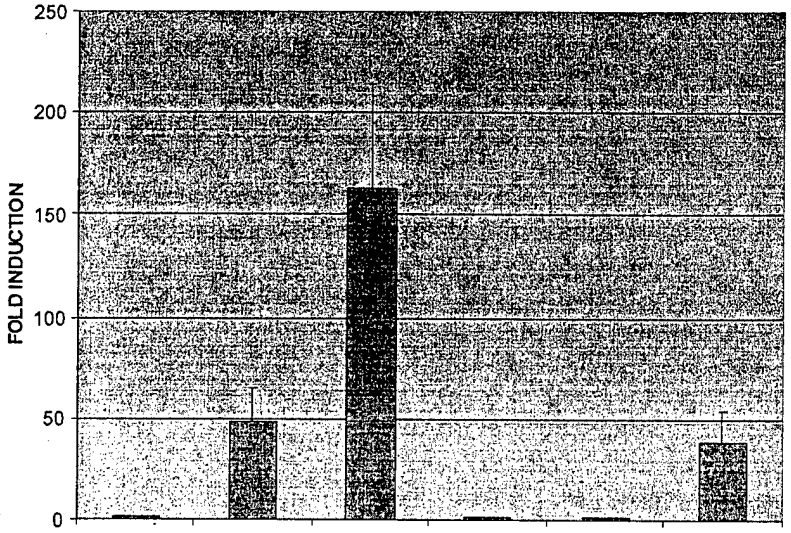
Task B. Mapping the β-Catenin Binding Region of AR

1. Determine the region of AR that interacts with β-catenin

1.1 *β-catenin can bind to and increase the transcriptional activity of AR and even restore the activity of truncated AR.*

AR contains three domains: a variable N-terminal domain involved in transcriptional activation, a DNA-binding domain, and a C-terminal ligand-binding domain (LBD). A unique property of the AR is the N/C interaction, which is an androgen-induced interaction between the NH_2-terminal domain and the carboxyl-terminal LBD (6,7). The N/C interaction requires the binding of agonists that have androgen activity in vivo. The high affinity natural androgens, dihydrotestosterone(DHT) and testosterone, and the synthetic agonists, mibolerone and methyltrienolone (R1881) at concentrations of 0.1–1 nM, induce the N/C interaction in mammalian two hybrid assays (8). To identify the area of AR that is responsible for the interaction with β-catenin we first used a truncated loss-of-function AR (507-919) expression plasmid that lacks the N terminal domain and hence, lacks the N/C interaction. This plasmid was compared to the full length AR in transfection assays with a β-catenin expression plasmid in the AR negative CV1 cells (Fig.1). The results show that β-catenin can increase the transcriptional activity of full-length AR three fold in the presence of DHT. As expected, because the NH2-terminal portion plays an important role

in AR's transcriptional activation, the truncated AR (507-919) lost its activity in the presence of DHT. However, when β-catenin was cotransfected with the truncated AR (507-919) the activity of this mutant was restored to 80% of the activity of wild type AR, suggesting that β-catenin interacts with the C-terminus of AR and mimic the function the N-terminus in stabilizing AR and potentiating its activity.



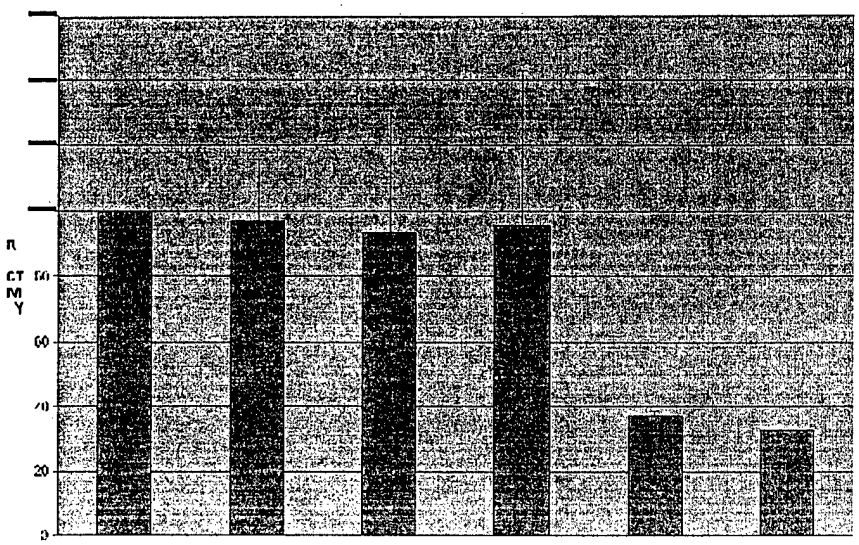
MMTV-LUC	+	+	+	+	+	+
WT AR	+	+	+	-	-	-
AR(507-919)	-	-	-	+	+	+
DHT(10 nM)	-	+	+	-	+	+
β-catenin	-	-	+	-	-	+

Fig. 1. β-catenin can bind to and increase the transcriptional activity of AR and even restore the activity of truncated AR. CV1 cells were cotransfected with 40ng of wild type AR(WT AR), 100 ng of MMTV-LUC, 400ng of β-catenin or 40ng of truncated AR(AR(507-919)) constructs or equimolar amount pcDNA expression vector. Data were from three independent experiments as relative activities normalized to protein level.

1.2. A point mutation in the AF2 domain of the AR inhibits its interaction with β-catenin.

In our previous report we showed that β-catenin can bind to the AR and function as an AR coactivator (5). Following our publication, another group confirmed our findings and showed that the ligand binding domain (LBD) of AR and the central region spanning the armadillo repeats 1-6 of β-catenin were involved in the interaction (9). We therefore focused our study in mapping more precisely the area in the LBD involved in the interaction. In order to investigate which amino acid in the LBD of AR is important in the interaction between AR and β-catenin, a panel of point mutants of AR were used. Mutations in the androgen receptor C terminus were chosen based on known changes that caused androgen insensitivity syndromes (I898T, K720A, I737T, V716R, E897K), as referenced in the androgen receptor database (http://ww2.mcgill.ca/androgendb/). We

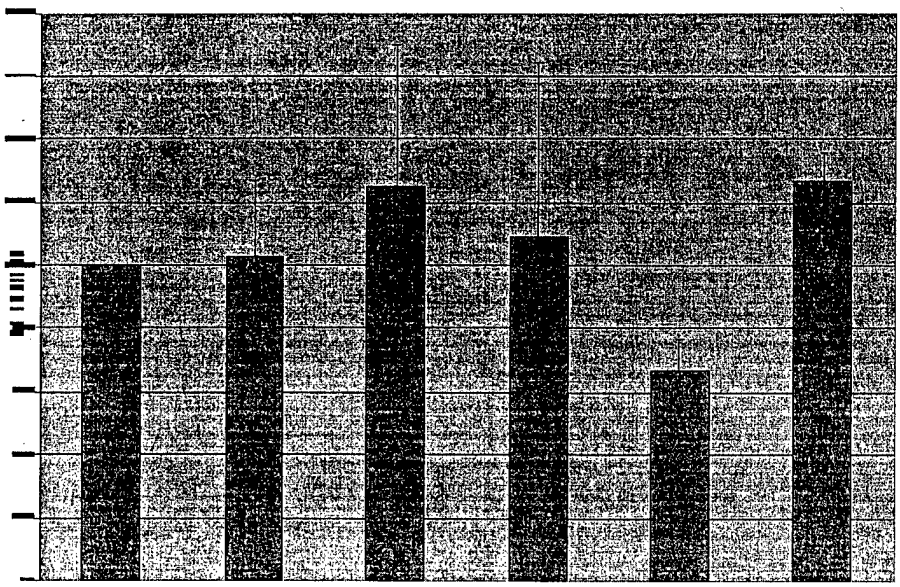
initially evaluated the transcriptional activity of these mutants in the LNCaP cells in the presence of DHT.



MMTV-LUC	+	+	+	+	+	+
DHT(10 nM)	+	+	+	+	+	+
AR	WT	ARI898T	ARK720A	ARI737T	ARV716R	ARE897K

Fig. 2. Effect of point mutants of the AF2 domain on AR inhibits transcriptional activity. CV1 cells were cotransfected with 40 ng of wild type AR (WT) or different mutants, 100 ng of MMTV-LUC constructs or equimolar amount pcDNA expression vector. Data were from three independent experiments as relative activities normalized to protein level.

Studies done by other investigators have shown that a mutation at aminoacid 720 (AR K720A) does not interfere with the N-C interaction but eliminates co-activator binding. Mutations at AR E897K and V716R are defective in the N/C interaction (6,7). We first analyzed these mutants obtained from Dr E. Wilson (University of N. Carolina, Chapel Hill) in an AR transcriptional assay. Our results confirm that both amino acid 716(V716R) and 897(E897K) are essential for transcriptional activity of AR, but not aminoacids 898, 737,720 (**Fig. 2**). AR activity was decreased to approximately one third when either of these amino acids were mutated. We then studied the effects of these mutants on the interaction with β-catenin by using cotransfections in CV 1 cells and measuring transcriptional activity induced by DHT. Surprisingly, only mutant V716R can decrease the effect of β-catenin on the transcriptional activity of AR (**Fig. 3**). Therefore while both amino acid 716 and 897 of AR play an important role in AR transcriptional activity, only amino acid 716 is important for the interaction between AR and β-catenin. Our results disagree with recent data published by Song et al (13). This group used a Gal4/AR LBD construct to study the interaction of the LBD with the NTD and β-catenin and showed that the Gal4/AR LBD mutant V716R was able to bind ligand but did not interact with AR NTD or TIF2. However, they found that Gal4/AR LBD(V716R) interacted with a VP16/β-catenin construct and concluded that this aminoacid was not essential for the interaction of β-catenin with AR but was necessary for interaction between the LBD and NTD.



MMTV-LUC	+	+	+	+	+	+
DHT(10 nM)	+	+	+	+	+	+
AR	WT	I898T	K720A	I737T	V716R	E897K
β-catenin	+	+	+	+	+	+

Fig. 3. A point mutation of the AF2 domain of the AR inhibits its interaction with β-catenin. CV1 cells were cotransfected with 40 ng of wild type AR(WT) or different AR mutants, 100 ng of MMTV-LUC, 400 ng of β-catenin constructs or equimolar amount pcDNA expression vector. Data were from three independent experiments as relative activities normalized to protein level.

We used a full length human AR with a V716R mutation and in our experiment this mutation significantly weakens the interaction with β-catenin compared with wild type AR. On the other hand, Song et al found that a GAL4 /AR LBD construct with a mutation at aminoacid 720 (K720A) reduced the binding to the AR NTD by 50% and completely abrogated binding to the VP16/β-catenin. We used a full length human AR with a K720A mutation and found that the effect of β-catenin on AR's transcriptional activity was unchanged, suggesting that these aminoacid does not participate in the interaction of β-catenin with AR. We believe our results reflect more reliably the physiological conditions since we employed a human full length AR and not a GAL4/AR LBD construct. It is possible that the conformational changes induced by the GAL4 domain may result in artificial findings. Additionally Song et al used a VP16/β-catenin construct instead of a pure β-catenin construct as we did in our experiment. VP16 itself has strong transactivation capabilities and may lead to artificial results. Our results support data published by Pawlowski et al who used a " nuclear shuttling" assay to evaluate the functional interaction between AR and β-catenin. They showed that AR K720A, AR 897K and V716R are all effective in translocating β-catenin to the nucleus and that a functional N-C interaction or p160 co-activator binding are not required for the interaction of AR/β-catenin.

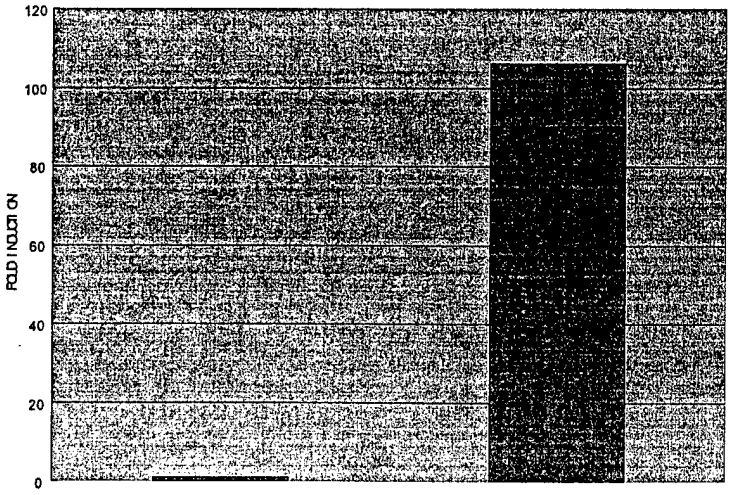
2. Do Beta Catenin and AR bind in Association with an Androgen Response Element?

We initially proposed to use gel mobility shift assays using purified proteins to investigate if β-catenin enhances the binding of AR to its cognate DNA recognition sequence. Recently the technique of chromatin immunoprecipitation (ChIP) has been developed and used as a powerful technique to recognize endogenous transcription factors assembled onto gene promoters in vivo. We decided to take advantage of this new assay to better understand AR-dependent transcription in vivo and the dynamics of AR/beta catenin interaction at gene promoters. The genes activated by AR often contain enhancer and promoter elements in their regulatory regions. This is the case for the gene encoding for the prostate-specific antigen (PSA), the best-characterized androgen-responsive gene in the prostate gland. The Chip Assay was performed using the LNCaP cells that contain an endogenous AR as well as the prostate-specific antigen gene (PSA). The human PSA promoter was chosen as the target promoter because androgen-induced PSA synthesis is a well-characterized event in LNCaP cells. Biochemical and genetic studies showed that both the enhancer and the promoter in the PSA gene display androgen responsiveness but a maximal activity requires the presence of both. The proximal promoter has been localized to a 630 bp fragment containing a core TATA box and two putative androgen-responsive elements (AREs) ARE 1, and ARE 2 (21). The AR activates transcription synergistically through these AREs. The enhancer element, centered at approximately 4.2 kb, is located within a 6 kb region (22) and harbors another putative ARE, termed ARE 3. It is yet unknown how the enhancer and the promoter, almost 4 kb apart, are able to coordinate the assembly of an AR transcription complex.

2.1. Transient loading of AR and recruitment of β-catenin and other coactivators to the PSA promoter and enhancer

Nuclear receptors influence the rate of transcriptional initiation though several mechanisms: they interact with the basal transcription machinery and also modify the state of chromatin organization at the promoter of target genes. The AF-2 domain of nuclear receptors is important for this activity as it allows the interaction with a variety of cofactors such as histone acetyltransferases or histone acetylases which lead to alterations in nucleosome structure (17,18). The rates of gene transcription roughly correlate with the degree of histone acetylation such that hyperacetylated regions of the genome are more actively transcribed than hypoacetylated regions (19,20).

Previous studies in fibroblast cell lines showed that the histone deacetylase inhibitor TSA (trichostatin A) can increase the transcriptional activity of AR induced by dihydrotestosterone (DHT) (23). We studied the role of histone acetylation in AR activity in the LNCaP prostate cancer cells. (**Fig. 4**) The results show that pretreatment with TSA augments the effects of DHT on AR-dependent transcription from the mouse mammary tumor virus promoter. Given the magnitude of the enhancement of AR activity by TSA (100 fold) it is clear that histone acetylation and chromatin remodeling play a crucial role in DHT-induced AR activity.



MMTV-LUC (100 ng)	+	+
AR (40 ng)	+	+
DHT (10 nM)	+	+
TSA (1 ng/ml)	-	+

Fig. 4. Inhibition of histone deacetylation increases the transcriptional activity of AR. CV1 cells were cotransfected with AR and MMTV-LUC expression constructs. Cells were treated for 24 hours with 10 nM DHT with or without pretreatment for 4 hours with 1ng/ml of TSA.

Studies done in the laboratory of Myles Brown showed that loading of holo-AR and recruitment of RNA polymerase II to promoters occurs transiently. The cyclical nature of the AR transcription complex assembly is also reflected in the transient association of the coactivators GRIP1 and CREB-binding protein and the acetylated histone H3 with the PSA promoter (14). However, to our knowledge, the recruitment of β-catenin in conjunction with AR to the target genes has not been studied. We therefore used chromatin immunoprecipitation to analyze in vivo the recruitment of β-catenin in the AR transcription complex to the PSA promoter and enhancer in vivo in LNCaP cells. The results shown in Fig. 6 confirm that β-catenin is indeed recruited in the AR transcription complex and that the loading of holo-AR and β-catenin to the promoter (ARE1) and enhancer (ARE3) of the PSA gene occur transiently. The timing of β-catenin's recruitment to the PSA gene is similar to that of AR in that both AR and β-catenin are recruited to the PSA promoter within 15 minutes following the addition of DHT and return to base line after approximately 60 minutes. At the PSA enhancer, loading of AR and β-catenin occurs in exactly the same pattern following the addition of DHT. The results indicate that the dynamic loading of β-catenin to the PSA promoter and enhancer shares the same pattern with AR, strengthening the hypothesis that β-catenin plays an important role in AR's transcription. Our data supports the findings of Mulholand et al. and Pawloswki et al. who used immunofluorescence to study the dynamics of the interaction between AR and β-catenin. Although Pawloski studied the interaction in neuronal cells and Mulholand in the LNCaP prostate cells, both groups had similar results: upon ligand addition (DHT), AR translocated to the nucleus and with it β-catenin also increased in the nucleus in a similar time fashion. In addition, Pawloski also showed that at least in the neuronal cells,

translocation of AR alone is not sufficient to induce complete nuclear localization of β-catenin and that agonist (DHT) but not antagonist-bound AR was required. They also showed that the AR antagonist bicalutamide was still able to translocate AR to the nucleus but failed to induce β-catenin translocation. (13) The fact that in our experiment β-catenin and AR shared similar timing in their recruitment to the PSA gene is consistent with the hypothesis that AR is responsible for β-catenin's translocation to the nucleus.

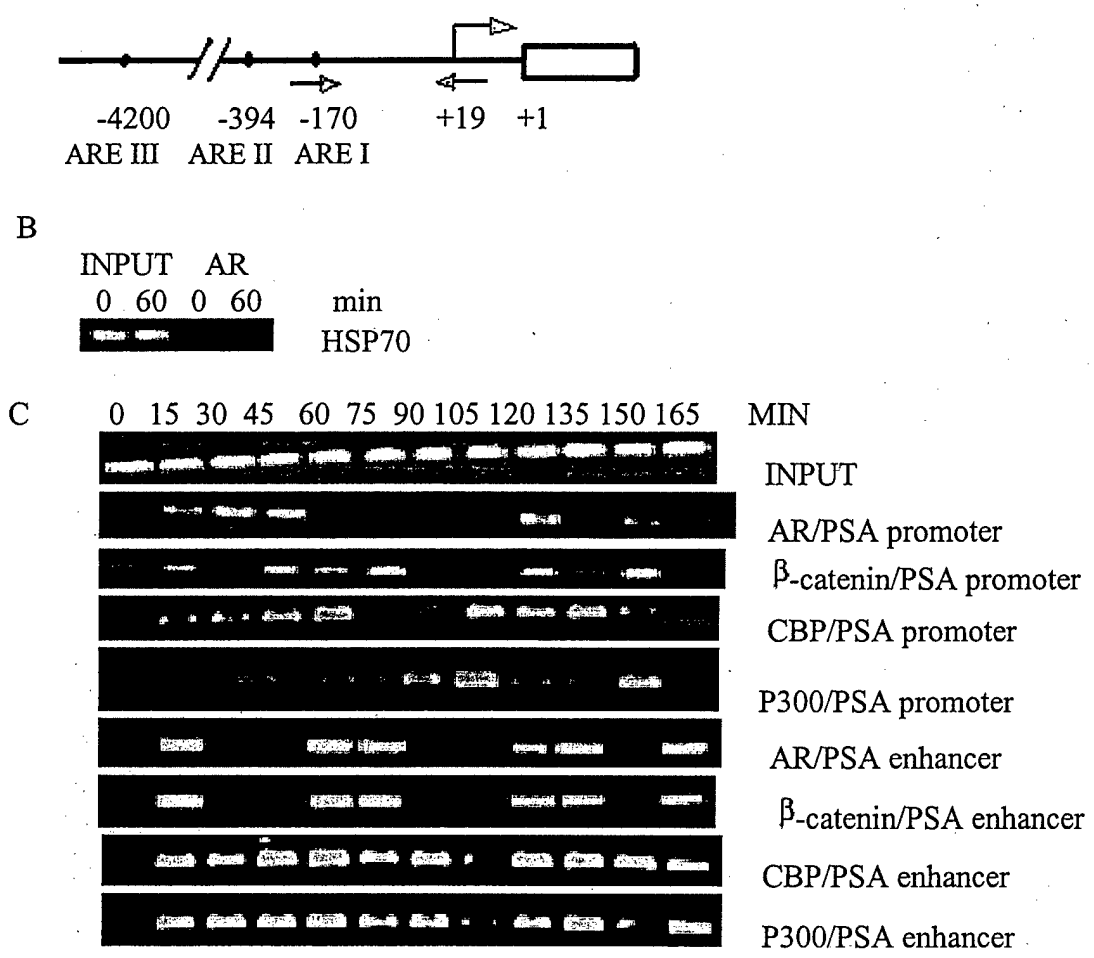
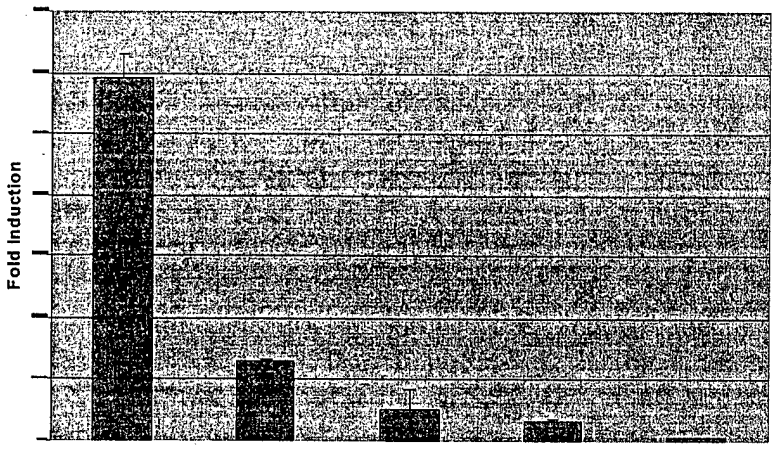


Fig. 5. Transient loading of AR and recruitment of β-catenin and other coactivator to the PSA gene promoter and enhancer in response to DHT treatment in LNCaP cells. *A*. Schematic representation of the PSA promoter region analyzed by the ChIP assay. The localization of the PCR primers (*arrow*) and androgen-response elements (*ARE*) is shown. *B*. Negative control of ChIP assay. LNCaP cells were treated with 10 nM DHT for 60 min before harvesting for ChIP assay. Chromatin samples were immunoprecipitated with anti-AR antibody and analyzed by PCR with *HSP70* gene-specific primers. *Input*, DNA prior to immunoprecipitation. C. LNCaP cells were incubated with 10 nM DHT for indicated times before harvesting for ChIP assay. Chromatin samples were immunoprecipitated with anti-AR antibody and specific antibodies to other coactivators, such as anti β-catenin antibody, prior to PCR with PSA gene promoter-specific or PSA gene enhancer-specific primers followed by agarose gel electrophoresis and ethidium bromide staining. *Input*, DNA prior to immunoprecipitation.

2.2 The effects of bicalutamide on recruitment of AR coactivators and corepressors to PSA promoter and enhancer

Although androgen ablation causes regression in the majority of cases of early stage prostate cancer, ultimately prostate cancer becomes androgen independent and refractory to hormonal manipulations either by androgen ablation or by antiandrogens (14,24,25). The mechanisms by which tumor cells escape androgen ablation and become independent of the need for androgens are not fully understood. The antiandrogens most commonly used in the clinic are the nonsteroidals flutamide and bicalutamide. While flutamide's active metabolite hydroxyflutamide has mild agonist activity as well, bicalutamide is a pure antagonist for both wild type as well as mutant AR and therefore has replaced flutamide as the most widely used antagonist in the clinic. However the mechanisms by which these antagonists inhibit AR are not completely understood. Previous studies showed that in the absence of ligand AR is complexed with a heat shock protein chaperone complex (HSP90). Upon ligand binding AR undergoes conformational changes, homodimerizes, translocates to the nucleus, binds DNA and activates transcription. Until recently it was thought that in the presence of bicalutamide AR remains in the cytoplasm associated with HSP90 and therefore unable to activate transcription. Newer data published by Masiello et al. shows that bicalutamide does not prevent AR's nuclear localization and in fact may even stimulate AR DNA binding. Their hypothesis is that bicalutamide inhibits the association of the AR N and C terminus or the interaction with steroid receptor coactivator proteins, namely SRC-1/2. This group showed that coactivator expression (SRC-1) increases the bicalutamide concentration required to antagonize AR transcriptional activity. Because of this new data we decided to investigate the role of bicalutamide (Casodex®) on the interaction between AR and β-catenin. In our experiments, using LNCaP cells, 5 uM bicalutamide can effectively inhibit the AR transcription induced by 10 nM DHT and 10 uM bicalutamide abolishes nearly 90% of AR activity (**Fig. 6**).



DHT (10nM)	+	+	+	+	+
Bicalutamide	0 uM	5 uM	10 uM	30 uM	100 uM
MMTV (100ng)	+	+	+	+	+

Fig 6. The effects of bicalutamide (Casodex) on AR activity. The LNCAP cells were transfected with 100 ng of MMTV reporter gene. 24 hours later, the cells were treated with 10 nM DHT and different levels of bicalutamide for another 24 hours and luciferase activity was measured.

In order to further investigate the mechanisms by which bicalutamide blocks AR-dependent transcription, we used the ChIP assay to assess the loading of AR to and endogenous androgen regulated gene (the PSA promoter and enhancer) in the presence or absence of bicalutamide. In addition, we also studied the recruitment of AR coactivators and corepressors (β-catenin, CPB, P300, SMRT and NCoR) in response to bicalutamide (**Fig. 7**). The results indicated that bicalutamide recruited both AR and β-catenin to the PSA promoter but not to the enhancer. What is more important, we found that bicalutamide can inhibit the loading of AR and β-catenin to the PSA promoter in the presence of DHT. (**Fig. 7 B**). This results indicate that bicalutamide inhibits AR activity in the presence of androgens by inhibiting the loading of the AR /β-catenin complex to the PSA promoter. This may explain why bicalutamide is not effective in prostate cancer patients who have not had androgen ablation since the levels of agonist (DHT) may be too high for bicalutamide to be able to inhibit the loading of AR. We also found that bicalutamide differentially recruits the coactivator p300, but not CBP to the PSA enhancer.

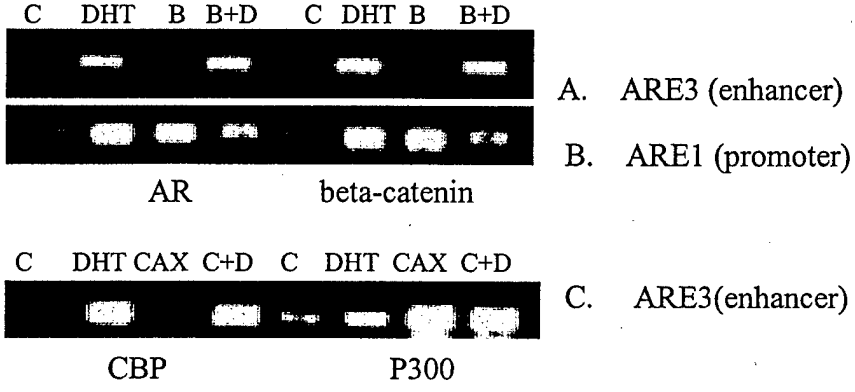


Fig. 7. The effects of bicalutamide (B) on the recruitment of AR and coactivators to PSA promoter and enhancer. LNCaP cells were incubated with 10 nM DHT in presence or absent of 10 uM bicalutamide for 45 minutes before harvesting for ChIP assay. Chromatin samples were immunoprecipitated with anti-AR antibody other specific antibodies prior to PCR with promoter-specific primers followed by agarose gel electrophoresis and ethidium bromide staining. C: Control, no treatment, DHA: 10 nM DHT, 45 min, bicalutamide: 10 uM bicalutamide, 45 min, B+D: 10 nM DHT plus 10 uM bicalutamide, 45 min.

In the case of the estrogen receptor (ER) the switch from gene activation to gene repression is thought to occur through antagonist binding. For example, antagonist-bound ER has been shown to interact with corepressors in vitro and to be associated with corepressors and HDACs in vivo. The structural and functional similarity between ER and AR lead to the hypothesis that a similar corepression complex may be recruited by antagonist-bound AR (19). We found that bicalutamide can recruit SMRT to both the promoter and enhancer and that even higher levels of SMRT were recruited to the PSA gene by bicalutamide in

the presence of DHT (**Fig. 8.**). Therefore our data supports a model in which bicalutamide acts as an AR competitive antagonist by inhibiting the loading of the AR/β-catenin complex to the promoter of the PSA gene and in addition, by recruiting AR corepressors to the PSA gene.

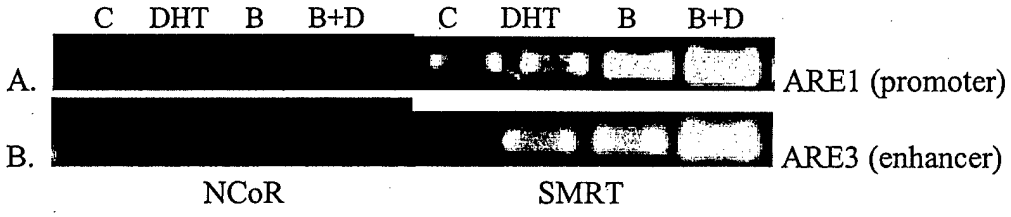


Fig. 8. The effects of bicalutamide on the recruitment of AR corepressors to the PSA promoter (A) and enhancer (B). LNCaP cells were incubated with 10 nM DHT in presence or absence of 10 uM bicalutamide for 45 minutes before harvesting for ChIP assay. Chromatin samples were immunoprecipitated with specific antibody against NCoR or SMRT antibody prior to PCR with promoter-specific primers followed by agarose gel electrophoresis and ethidium bromide staining. C: Control, no treatment, DHT: 10 nM DHT, 45 min, B: 10 uM bicalutamide, 45 min, B+D: 10 nM DHT plus 10 uM bicalutamide, 45 min.

Task C. Determine if E-cadherin competes with AR for binding to β-catenin

The functional roles of β-catenin in adhesion and signaling are made possible by a precise subcellular compartmentalization of the protein in at least two distinct, but interchangeable pools. One pool is located at the cell membrane, where β-catenin forms a complex with E cadherin. E-cadherin is a calcium-dependent transmembrane protein that mediates cell-cell adhesion. A second, E-cadherin independent pool of β-catenin is in the cytoplasm and is essential for signaling. Recently, binding of E-cadherin to the cytosolic pool of β-catenin has been shown to decrease β-catenin transcriptional activity, supporting a role for E-cadherin as a regulator of β-catenin signaling (10,11). In prostate cancer, a major fraction of tumors have decreased or absent expression of E-cadherin, which correlates with high tumor grade and poor prognosis in prostate cancer patients (12). These findings imply that the increased availability of β-catenin resulting from the loss of E-cadherin expression could contribute to increased androgen-dependent transcription and accelerated tumor progression. We investigated the physiological interaction among AR, β-catenin and E-cadherin in the AR positive LNCaP cells by immunoprecipitation. The results shown in **Fig. 9** indicate that while AR can bind to β-catenin in the presence of DHT, AR does not bind E-cadherin. This suggests that AR and E-cadherin may occupy the same domain of β-catenin, therefore β-catenin can only interact with AR or E-cadherin, but not both. In this case, E-cadherin may compete with AR for binding to the same domain of β-catenin and prevent the interaction between AR and β-catenin, thus inhibiting the AR activity. We are planning to map more precisely the domain of β-catenin that is shared by AR and E-cadherin.

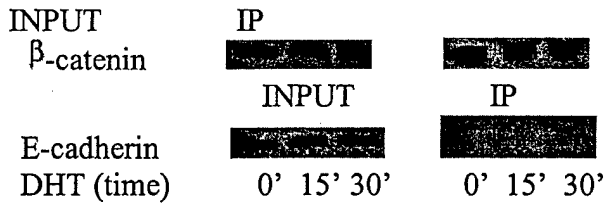


Fig. 9. AR can bind to β-catenin but not E-cadherin. LNCaP cell lysates were immunoprecipitated (IP) with anti-AR and immunoblotted with anti-β-catenin or anti-E-cadherin to detect the binding between AR and β-catenin or AR and E-cadherin.

Task A. Determine the Region of β-catenin that interacts with the Androgen Receptor

Since the time we submitted our proposal to study the interaction between β-catenin and AR a number of papers have been published on this subject and this aim has been extensively studied by other investigators and published. Yang et al used a yeast-two hybrid system to identify proteins that interact with a construct containing the LBD and hinge region of AR and identified the known coactivators SRC1 and ARA 70 as well as β-catenin (16). They also showed that β-catenin selectively binds to AR but not to other steroid receptors such as the estrogen receptor, the progesterone receptor and glucocorticoid receptor (9). The ligand binding domain of AR (LBD) and the central region spanning the armadillo repeats 1-6 of β-catenin were found to be responsible for the interaction. To precisely map the interacting region in β-catenin they used a yeast two-hybrid assay with series of truncated mutants of the protein in which each single armadillo repeat was subsequently deleted. While deletion of repeats 7-12 showed about two-thirds of the activity of the full-length protein, deletion of repeat 6 essentially abolished the interaction with AR indicating that this repeat is crucial for the biding to AR. They further used a PCR-based site-directed mutagenesis technique to further confirm that the region spanning armadillo repeats 1-6 is mainly responsible for binding to AR.

C. KEY RESEARCH ACCOMPLISHMENTS

- β-catenin can increase transcriptional activity of AR and can even restore the activity of truncated AR
- Aminoacid 716 is essential for the interaction between β-catenin and AR and mutation of this aminoacid results in loss of the interaction between the two molecules
- β-catenin is recruited in the AR transcription complex to the promoter of the PSA gene (prostate specific antigen gene) and the loading of holo-AR and β-catenin to the promoter and enhancer occurs with remarkable similar timing, within 15 min following addition of DHT, returning to baseline after 60 min
- the antiandrogen bicalutamide recruits both AR as well as β-catenin to the PSA promoter but NOT the enhancer and it can inhibit the loading of AR/β-catenin to the PSA promoter in the presence of DHT. Bicalutamide also recruits the Ar corepressor SMRt to the PSA promoter and enhancer.

- AR binds to β-catenin but Not to E cadherin suggesting that β-catenin can interact either with AR or E cadherin but not with both simultaneously

D. Conclusions and Recommended Changes/Future Work

Data published in recent years and data included in this report indicate that β-catenin plays an important role in regulating the AR transcriptional activity and could be a key player in the progression of prostate cancer to androgen independence. Several investigators have published on the minimal necessary components of AR and β-catenin for the interaction. Yang et al. used a yeast two hybrid assay with AR LBD as a bait and identified β-catenin as an interacting protein. They showed that the armadillo domain of β-catenin and more specifically repeat 6 is essential for the interaction and showed that β-catenin can translocate to the nucleus as part of a complex with AR by an interaction thorugh armadillo repeat 6. They also showed that E cadherin modulates the level of cytoplasmic pools of β-catenin to enhance AR-mediated transcription. They used an AR negative E cadherin negative cell line (TSU.pr1) and a stable transfectant expressing E cadherin. They showed that E cadherin in the stable transfectants induced a redistribution of the cellular localization of β-catenin which directly affected AR-mediated transcription. Mulholland et al. used AR expressing cells (LNCaP) and AR negative cells (PC3) for time course cell fractionation experiments and showed that AR can shuttle β-catenin into the nucleus when exposed to androgens. They also showed that in the presence of androgen AR and β-catenin show distinct punctate colocalization in the nucleus. They also used a DNA binding assay (acrydite assay) to show that β-catenin binds the probasin promoter in an AR-dependent manner. Another paper by Song et al also recently studied the AR/β-catenin interaction and showed that β-catenin binds the AR LBD and modulates the transcriptional effects of TIF2 and the N-terminal domain of AR (NTD). Therefore we conclude that the aims proposed in our initial application have been addressed thoroughly in the literature and confirmed by several investigators, firmly establishing that the LBD of AR and the armadillo region of beta catenin are responsible for the interaction between the two proteins. Further pursuing the original aims of our proposal will not result in an original contribution to our understanding of the role of β-catenin in AR signalilng.

None of the previously published studies have investigated the effect of beta catenin down regulation on AR function. In general, mammalian genetic approaches to study gene function have been hampered by the lack of tools to generate stable loss-of-function phenotypes efficiently. In a recent paper, Agami et al. reported a new vector system, pSUPER, which directs the synthesis of small interfering RNAs (siRNAs) in mammalian cells (26). They showed that siRNA expression mediated by this vector causes efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time (26). In order to generate stable β-catenin negative prostate cancer cell, we are taking advantage of this new vector system to downregulate β-catenin levels.

Task 1. Investigate the effects of loss-of-function of β-catenin on the LNCaP Prostate cells

i. Establish the loss-of-function of beta catenin phenotype in LNCaP cells by using the siRNA technique.
ii. Evaluate the effects of loss-of-function of beta catenin on the AR activity and DNA remodeling.
iii. Evaluate the role of beta catenin mutants found in prostate cancer patients on the development and progression of prostate cancer.

Task 2. Mechanisms involved in the development of androgen independent (AI) prostate

Continuing our study on the effect of bicalutamide on the AR and of mechanisms involved in the development of the androgen independent status, we plan to take study a pair of androgen-dependent and independent prostate cells already published in the literature (Leland W.K. Chung PhD, Emory University)

i. We will use western blot to examine the levels of AR, beta catenin and other AR cofactors in the paired androgen dependent and independent (AI) LNCaP cells
ii. We will use the ChiP assay to study the loading and recruitment of AR and AR cofactors to AREs in the androgen independent cells as compared to the androgen dependent cells.
iii. Investigate the effects of bicalutamide on the loading of AR and the recruitment of AR cofactors to the promoter and enhancer of PSA gene in the androgen independent cells.
iv. Investigate the effects of knocking out beta catenin on the sensitivity to antiandrogens in the androgen independent LNCaP cell.

Task 3. Determine if E-cadherin and AR compete to binding the same domain of β-Catenin

i. Determine if binding of β-catenin to E-cadherin prevent the interaction between AR and β-catenin
ii. Use immunofluoresce to investigate the interaction pattern among AR, β-catenin and E-cadherin.
iii. Determine the binding domain of β-catenin shared by AR and E-cadherin and investigate the effects of different β-catenin mutants on this interaction.

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